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Establishment of Hamster Blastocyst-Derived Embryonic Stem (ES) Cells

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The establishment of four ES cell lines from the Syrian "golden" hamster (*Mesocricetus auratus*) is described. The cells can be maintained in the undifferentiated state when grown on primary mouse embryonic fibroblast feeder layers. In suspension culture they spontaneously differentiate into embryoid bodies of increasing complexity which contain a variety of tissues including embryonic ectoderm and myocardium. All four lines—one female and three male—are karyotypically normal with 44 chromosomes. Hamster is the second species from which ES cells have been established. As in mouse, the cells should be useful for developmental and transgenic studies.

INTRODUCTION

The usefulness of ES cells (Evans and Kaufman, 1981; Martin, 1981) has now been demonstrated at both the *in vitro* and *in vivo* levels. The pluripotency of ES cells in culture (Doetschman *et al.*, 1985) has been used to provide insights into embryonic vasculogenesis and angiogenesis (Risau *et al.*, 1988). The totipotency of ES cells carrying a genetic alteration (Gossler *et al.*, 1986; Robertson *et al.*, 1986) has been used to create mouse genetic equivalents to the human Lesch-Nyhan syndrome (Hooper *et al.*, 1987; Kuehn *et al.*, 1987). These characteristics, coupled with the ability to use homologous recombination between incoming DNA and chromosomal genes to correct (Doetschman *et al.*, 1987) or mutate (Thomas and Capecchi, 1987; Doetschman *et al.*, in preparation) specific genes in ES cells, provide precision tools to alter endogenous genes in a predetermined way. As a result, new ways to study gene regulation and function in both embryo and adult, and models for human diseases, can be expected.

The establishment of ES cell lines from species other than mouse should be of considerable value because it would allow one to choose the experimental animal most appropriate for any given problem. In several cases, hamsters are more suitable than mice. For example, there are hamster strains which have cardiomyopathy (Homburger, 1979), a disorder which may be manifested at the stage of ES cell *in vitro* differentiation when myocardium is first formed. In addition, because the lipid metabolism of the hamster has more similarities to that of man than mouse (Spady and Dietschy, 1985), transgenic hamsters made from ES cells carrying mutations affecting lipid metabolism would be useful for investigating atherogenesis. In this paper we describe the establishment and maintenance of hamster ES cell lines and show that they are highly pluripotent.

MATERIALS AND METHODS

Establishment and maintenance of hamster ES cells. Eight to twelve-week-old Syrian "golden" hamsters (Harlan Sprague-Dawley, Indianapolis) were superovulated with 20 units of pregnant mare serum the morning after ovulation. Three and one half days later each female was placed overnight with three males. Blastocysts were flushed from the uterus 3 days and 5 hr postfertilization (Bavister *et al.*, 1983). The zona pellucida, which still remained on about one-third of the blastocysts, was removed by treatment with 0.5% pronase in PBS for several seconds. The blastocysts were placed into four-well tissue culture dishes (Nunc) in ES cell culture medium (Doetschman *et al.*, 1985) on a feeder layer of mouse primary embryonic fibroblasts prepared as described below. The blastocysts attached to the feeder cells 2 to 3 days later. The inner cell masses were mechanically disrupted into clumps of about 10 cells and passaged onto new feeders using micropipets. The resulting ES cell colonies were expanded in a similar manner until at least 50 colonies existed. Subsequently, a buffered trypsin/EDTA solution (0.04% trypsin, 115 m*M* NaCl, 4.5 m*M* KCl, 0.1% glucose, 3.5 m*M* $NaHCO_3$, 0.6 m*M* EDTA, pH 7.0) was used to passage the cells. The cells were not single-cell subcloned because it has been demonstrated by Martin (1981) that each individual cell in mouse ES cell lines derived from one blastocyst inner cell mass was pluripotent.

Embryonic fibroblasts were prepared from 14- to 16-day-old embryos. Five to ten embryos with liver and heart removed were minced and washed in PBS; trypsinized for 1 hr in trypsin/EDTA solution (0.5 hr, 25 ml; 0.5 hr, additional 25 ml) while being stirred; diluted 3:1 in 10% fetal calf serum in DMEM; sieved; centrifuged; plated in one 80-mm tissue culture dish per embryo,

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nedium changed to 5% fetal calf serum in DMEM the next day; expanded 3 to 5 passages (trypsin/EDTA); and irradiated (3000 rad) when at a confluent density.

Differentiation of hamster ES cells in suspension culture. ES cells growing on feeder layers were trypsinized, resuspended in standard medium, and allowed to sediment for 1 hr in the original culture dish to allow fibroblasts to preferentially adhere. The unattached ES cells were plated onto petri dishes in standard medium. The medium was completely changed every second day; on the other days it was supplemented with 0.5 vol of fresh medium. On the sixth and subsequent days the serum content of the medium was kept at 20% instead of 15%.

Chromosome count of hamster ES cells. ES cells were treated for 3 hr with 10 μg/ml of Colcemid, 30 min with 0.56% KCl, and 4 × 20 min with methanol/acetic acid (3:1; 4°C) before being spread onto microscope slides. The golden hamster karyotype (36 metacentrics; 8 acrocentrics) has been described by Galton and Holt (1964).

RESULTS AND DISCUSSION

In 10 experiments, 409 zona pellucida-free blastocysts from a total of 31 superovulated hamsters were brought into culture. Two to three days later, 233 blastocysts attached and 51 inner cell masses appeared (see Fig. 1A). From these, four cell lines (ES-Ma1, ES-Ma2, ES-Ma3, and ES-Ma4, each derived from an independent inner cell mass) were established (see Fig. 1B for an example). Thus, 1% of the blastocysts brought into culture produced ES cell lines. This compares to an average of 10% for mouse ES cells (Doetschman *et al.*, 1985). The lower percentage for hamsters may lie partly in the fact that the culture conditions used were established for mouse embryonal carcinoma and ES cells, there being no equivalent cell system for hamster. Although optimal conditions for culturing preimplantation hamster embryos have been described (Carney and Bavister, 1987), nothing is known about the medium requirements for early postimplantation hamster embryos, which are the conditions (in mouse, at least) required for ES cell culture. Now that hamster ES cell lines have been established, it should be possible to find more suitable culture conditions for them.

In establishing hamster ES cell lines, two difficulties had to be overcome. First, the inner cell mass cells had to be prevented from differentiating into round endoderm cells. This was achieved by frequent mechanical passaging as described under Materials and Methods. The second difficulty was finding out how to expand the ES cell colonies from just a few to a number (at least 50) which could display the synergistic rapid growth after trypsinization that is characteristic of ES cells. We

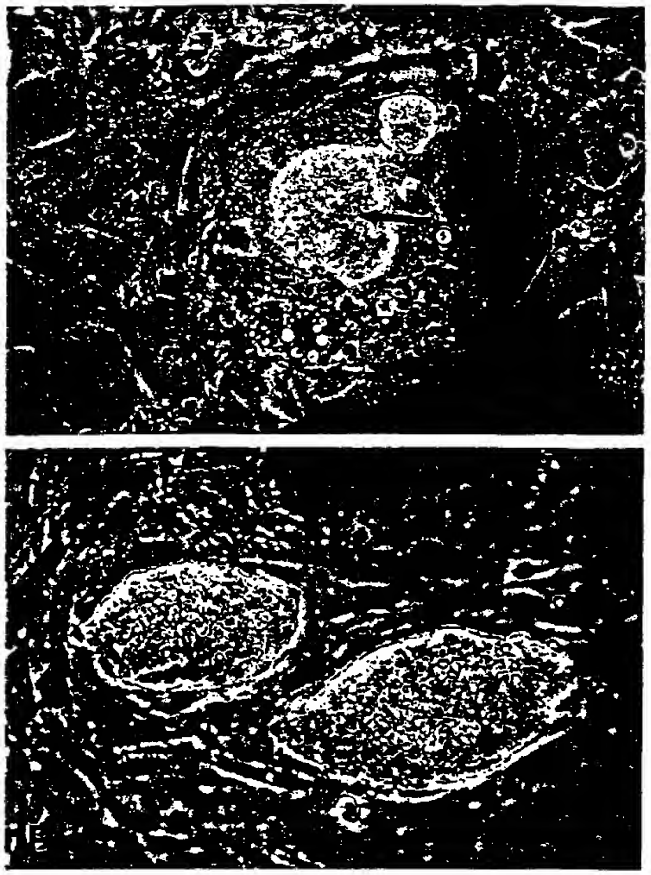

FIG. 1. Establishment of hamster ES cell lines. (A) A hamster blastocyst with inner cell mass cells (arrow) and trophectoderm cells (arrowhead) attached to a lawn of irradiated mouse, embryonic fibroblast feeder cells. (×125). (B) ES cells from the established hamster ES cell line ES-Ma1 also growing on feeder cells. (×200).

solved this problem by leaving the colonies undisturbed even though peripheral cells in the colonies differentiated into flat endoderm cells. We allowed this simultaneous growth and differentiation to continue as long as the growth of the undifferentiated cells outweighed their differentiation. We then passaged the undifferentiated ES cells mechanically so as to avoid the losses associated with trypsinization. In this way the number of undifferentiated ES cells was increased and expansion to the ~50 colony stage was achieved. At the 50 colony stage, passaging by trypsinization was commenced and differentiation could then be minimized by frequent passaging.

Hamster ES cell lines can be maintained in culture on a feeder layer of mouse embryonic fibroblasts by trypsin passaging every 2 to 3 days. Mouse fibroblasts appear to inhibit spontaneous hamster ES cell differentiation better than hamster fibroblasts. Hamster ES cells can be frozen and thawed, have been cultured now

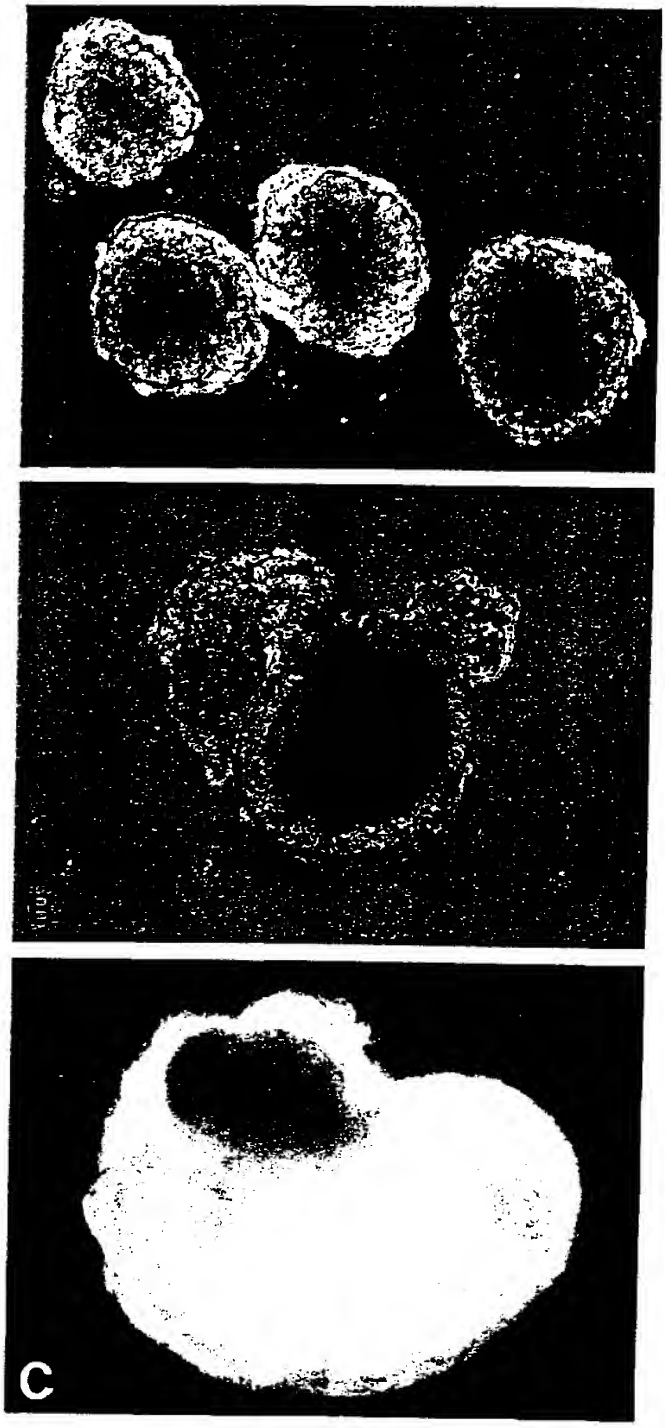

FIG. 2. Pluripotency of hamster ES cell line ES-Ma2. (A) Simple embryoid bodies at Day 3 of suspension culture. Note the outer layer of endoderm cells. (×125). (B) A complex embryoid body at Day 4 of suspension culture. Note the U-shaped layer of columnar ectoderm cells. (×125). (C) A cystic embryoid body at Day 16 of suspension culture. Dark-field optics. (×50).

for over 3 months without loss of undifferentiated state, and appear to be as immortal as mouse ES cells.

One of the distinguishing characteristics of mouse ES cells is that they manifest pluripotency when grown in suspension culture in the absence of feeder cells (Doetschman *et al.*, 1985). Since the degree of pluripo-

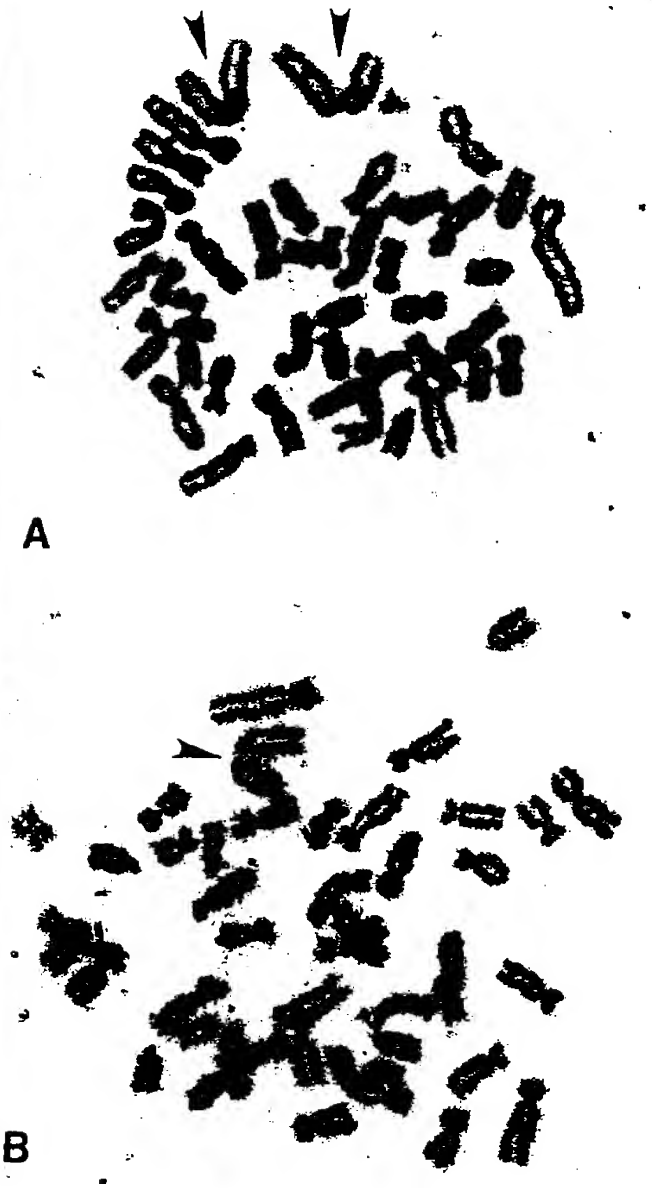

FIG. 3. Chromosome spreads of two hamster ES cell lines. (A) Female chromosome spread from ES-Ma1. (B) Male chromosome spread from ES-Ma2. Arrowheads indicate X chromosomes. The chromosome above the X chromosome in (B) is probably the Y chromosome (the Y chromosome is difficult to identify, Galton and Holt, 1964). (×1000).

tency of hamster ES cells will probably have practical importance only for *in vitro* studies, it was deemed unnecessary to induce tumors in athymic mice. Therefore, in order to determine the differentiation potential of the hamster ES cells, they were cultured in suspension. After 2 to 4 days, simple embryoid bodies were formed consisting of an outer layer of endoderm cells and an inner mass of undifferentiated cells (Fig. 2A). By 6 days most of the embryoid bodies had become more complex, with layers of columnar ectoderm cells (Fig. 2B), as in the egg cylinder stage of embryonic development. About 20% of these structures contained beating heart tissue by Day 9. After 14 days in suspension culture approximately half of the embryoid bodies had become cystic, a characteristic indicating the presence of visceral yolk sac endoderm (Fig. 2C). Occasionally, twitch contractions characteristic of skeletal muscle were seen, as well as keratin swirls. With the exception of keratin swirls, all of these tissues and cell types have been observed in mouse ES cell-derived embryoid bodies (Doetschman *et al.*, 1985; unpublished skeletal muscle cell observations), although at slightly different frequencies. Tissues observed in the mouse system but not detectable here are visceral yolk sac blood islands and smooth muscle (Doetschman *et al.*, 1985) and nerve cells (unpublished observations).

All four hamster ES cell lines appear to have a normal chromosome count. From 12 to 16 mitotic spreads were analyzed for each cell line and in each case 2 spreads were found to have less than 44 chromosomes. None of them contained more than 44 chromosomes. One of the lines, ES-Mal, is female (Fig. 3A); and the other three (see Fig. 3B) are male. Each of these lines had been in culture for at least 25 passages and all had been frozen and thawed at least once before chromosome analysis. These data are comparable to those derived from mouse ES cell lines (Doetschman *et al.*, 1985). Since G-banding has not been done we cannot be certain that the cell lines are euploid as are mouse ES cells.

The normal chromosome complement and the *in vitro* differentiation potential of hamster ES cells will provide a second ES cell system for developmental embryology and developmental gene function studies. We are presently carrying out experiments to determine if the hamster ES cells can colonize the germ line when introduced back into hamster blastocysts. If this proves to be possible, there will then exist a second ES cell system for small animal disease modeling.

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